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Anticoccidial Effects of Xanthohumol

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SUMMARY. Xanthohumol (XN), a prenylated chalcone from the hops flower, was examined for its ability to reduce invasion of Madin–Darby bovine kidney (MDBK) cells by *Eimeria tenella* sporozoites (SZ), as well as to reduce invasion by *E. tenella* and *E. acervulina* SZ in the chick host. Additionally, XN was tested as an anticoccidial feed additive at 20 ppm against challenge infections with *E. acervulina*, *E. maxima*, and *E. tenella*. Cell invasion by *E. tenella* SZ was inhibited 66% by treatment of SZ with 22 ppm XN. This inhibition was associated with an apparent physical disruption of the apical ends of the SZ. Rectal challenges with *E. tenella* SZ treated with 5, 10, and 20 ppm XN resulted in significantly reduced gross-lesion scores and normal chick-host weight gains compared with challenge with untreated SZ. Oral challenges with similarly treated *E. acervulina* SZ, accomplished with prior antacid treatment, resulted in significantly reduced gross lesions and reduced oocyst shedding compared with challenge with untreated SZ and were associated with physical disruption of sporozoite morphology. In a pilot test, provision of feed supplemented with 20 ppm XN for 3 days before challenge to 6 days after challenge did not control challenge infections with *E. acervulina*, *E. maxima*, or *E. tenella* as judged by measurements of weight gain, feed conversion, and gross lesions. Although XN-fed chicks infected with *E. acervulina* and *E. maxima* shed fewer oocysts than those on control feed, the differences in numbers were not statistically significant ($P > 0.05$).

RESUMEN. Efectos anticocidiales del Xantohumol.

El xantuhumol es un compuesto proveniente de la flor del lúpulo que fue examinado en su capacidad de reducir la invasión de esporozoitos de *Eimeria tenella* en células Madin-Darby de riñón bovino, así como de reducir la invasión de esporozoitos de *E. tenella* y *E. acervulina* en el ave. Adicionalmente el xantuhumol se evaluó como un aditivo anticoccidial en el alimento a una concentración de 20 partes por millón contra un desafío con *E. acervulina*, *E. maxima* y *E. tenella*. El tratamiento de esporozoitos con 22 partes por millón de xantuhumol inhibió la invasión celular por esporozoitos de *E. tenella* en un 66%. Esta inhibición se asoció con una aparente disrupción física de los extremos apicales de los esporozoitos. Desafíos rectales con esporozoitos de *E. tenella* tratados con 5, 10 y 20 partes por millón de xantuhumol, resultaron en reducciones significativas de los valores de lesiones y ganancias de peso normales en las aves, en comparación con el desafío con esporozoitos no tratados. Desafíos orales con esporozoitos de *E. acervulina* tratados con 5, 10 y 20 partes por millón de xantuhumol (previo tratamiento antiácido), resultaron en una reducción significativa de las lesiones macroscópicas y en una reducción de la diseminación de ooquistes en comparación con el desafío con esporozoitos no tratados. Los resultados se asociaron con la disrupción física de la morfología de los esporozoitos. En una prueba piloto, la administración de alimento suplementado con 20 partes por millón de xantuhumol durante tres días previos y 6 días posteriores al desafío, no se controlaron las infecciones con *E. acervulina*, *E. maxima* y *E. tenella* según se determinó por las ganancias de peso, la conversión alimenticia y las lesiones macroscópicas. Aunque las aves infectadas con *E. acervulina* y *E. maxima* alimentadas con xantuhumol diseminaron menos ooquistes que las aves alimentadas con el alimento control, las diferencias numéricas no fueron estadísticamente significativas ($P > 0.05$).

Key words: coccidiosis, *Eimeria*, chickens, hops, prenylated chalcones

Abbreviations: ATCC = American Type Culture Collection; GRAS = generally recognized as safe; GSH = glutathione; HBSS = Hanks' balanced salt solution; MDBK = Madin–Darby bovine kidney; MEM = minimum essential medium; PAS = periodic acid-Schiff; PBS = phosphate-buffered saline; SZ = sporozoites; XN = xanthohumol

Xanthohumol (XN) (3′-[3,3-dimethyl allyl]-2,4,4′-trihydroxy-6′-methoxychalcone) is the major prenylated flavonoid of the female flower of the hops plant (*Humulus lupulus* L.) (26). Hops have long been used in folk medicine and are commonly employed in the brewing industry to flavor beer. Additionally, hops are known to have preservative properties (13). A number of recent studies have focused on the biological activities of hops constituents and have revealed an array of beneficial attributes for XN, including antimicrobial activity (2,13), anticancer activity (15), inhibitory activity of specific enzymes such as induced nitric oxide synthase (31), UDP-glucuronosyltransferases (26), diacylglycerol transferase, (30) and triglyceride and apolipoprotein B secretion in liver cell culture (4). Of prime relevance to this article are reports (10,17) showing that XN inhibited growth of the apicomplexan parasite, *Plasmodium falciparum*, in a cultured red blood cell system, and additionally inhibited glutathione (GSH)–dependent hemin degradation by this parasite (10). Because coccidian parasites of the genus *Eimeria* are also apicomplexans, it seemed logical to test XN for activity against avian *Eimeria* as part of a program seeking alternative methods to control avian coccidiosis. Here described is the first report of anticoccidial activity of XN as determined by several experiments using both *in vitro* and *in vivo* assays.

MATERIALS AND METHODS

Chickens. Chickens used in these experiments were male, crossbred (White Rock male × Rhode Island Red female) birds, commonly referred to as sexsals, obtained as day-old chicks (Moyer's Hatchery, Quakertown, PA). They were raised under constant lighting and provided with free access to water and broiler starter ration, except briefly where indicated.

Parasites. Laboratory strains of *E. acervulina* (strain 12), *E. maxima* (Tyson's strain), and *E. tenella* (WLR strain) were used in these experiments. These species parasitize, respectively, the duodenum, mid-small intestine, and ceca, and have been maintained at this labo-

Table 1. Effect of 22 ppm xanthohumol treatment on MDBK cell invasion by *E. tenella* SZ. Counts of 50 fields at 1000×.[AB]

Treatment	SZ Inside cells	SZ Outside cells	% Invasion (inside/total × 100)	% Control (invasion)
Control	576	127	81.9	100
20 pm XN	76	144	34.5	34

[A]HBSS = Hanks' balanced salt solution; MDBK = Madin–Darby bovine kidney; SZ = sporozoites; XN = xanthohumol.

[B]Freshly prepared SZ (40,000/ml) were incubated with either 0 or 22 ppm XN in HBSS for 1 hr, centrifuged, then resuspended in culture medium and added to layers of MDBK cells on coverslips. After 2 hr at 41 C in 5% CO_2, culture fluid was removed and cell layers were washed, fixed in methanol, stained with HEMA-3, and examined for invasion by SZ.

ratory through serial passage in chickens every 3–5 mo. Stocks of oocysts have been stored routinely in 2% potassium dichromate at 4 C.

Preparation of SZ. Oocysts were freed of dichromate through repeated washings with tap water and centrifugation. They were further cleaned by incubating pelleted oocysts with three times the pellet volume of commercial bleach (to 6% sodium hypochlorite) on ice for 10 min. They were then floated from the remaining debris by centrifugation (1730 × *g*, 15 min). The oocysts were removed from the tops of tube contents, rinsed free of hypochlorite by repeated washings with tap water, and pelleted. The oocysts were cracked by vortexing pellets with equal pellet volumes of 1 mm glass beads. Excysting fluid (0.25 g trypsin [T 8128, Sigma, St. Louis, MO] and 0.75 g sodium taurodeoxycholate [T 0266, Sigma] in 100 ml Hanks balanced salt solution [HBSS, Sigma H 4891, which contains 0.1% D-glucose]) was added, and the SZ were excysted from the liberated sporocysts during incubation in a shaking water bath at 41 C for 15 min (*E. acervulina*) or 90 min (*E. tenella*). The liberated SZ were suspended in HBSS, were freed from debris by passing them through cellulose filter pads (11), and were washed twice in HBSS.

Xanthohumol. An approximately 85% pure preparation of XN was obtained as a yellow crystalline solid (S. S. Steiner, Inc., New York, NY) through the courtesy of G. Haas, Fairleigh-Dickenson University. A stock solution of 20 mg/ml was prepared in dimethylsulfoxide, and stored frozen. For use, the stock solution was diluted in HBSS to desired concentrations that ranged from 0 to 22 ppm as calculated on the dry weight of the XN preparation.

Treatment of SZ. Freshly prepared SZ were suspended in HBSS containing concentrations of XN ranging from 0–22 ppm. The concentration of SZ per milliliter of treatment depended upon the experiment. For a cell invasion study, *E. tenella* SZ were suspended at a concentration of 40,000/ml and treated with 0 or 22 ppm XN. For *in vivo* assays, *E. tenella* SZ were suspended at a concentration of 80,000/ml (equivalent to 10,000 sporulated oocysts) and treated with XN concentrations ranging from 0–20 ppm. *Eimeria acervulina* SZ were suspended at 11 × 10^6/ml (equivalent to about 1.4 × 10^6 sporulated oocysts) and treated with XN concentrations ranging from 0–20 ppm. SZ suspensions were treated by incubation for 1 hr at 40 C (body temperature of chickens) in a shaking water bath. They were spun-down and resuspended in equivalent volumes of culture medium (cell invasion) or HBSS (*in vivo* studies).

Table 2. Results from trial 1. Effects of cecal challenge with 80,000 XN-treated *E. tenella* SZ per chick on weight gains and gross lesion scores at 6 days postchallenge.[A]

SZ treatment group	Chicks per group	XN (ppm)	Gain[BC] (g)	Gross lesion score[BC]
Untreated/unchallenged	5	0	148 ± 8 a	0 ± 0 b
Untreated/challenged	5	0	135 ± 4 a	2.6 ± 0.5 a
1	5	20	152 ± 7 a	0 ± 0 b
2	5	2	139 ± 11 a	2.6 ± 0.7 a
3	5	0.2	134 + 5 a	2.0 + 0.5 a
4	5	0.02	145 + 15 a	1.2 + 0.5 a

[A]ppm = parts per million; SZ = sporozoites; XN = xanthohumol.

[B]Values are means ± standard error of the mean (SEM).

[C]Within columns, means with no common letter, differ significantly ($P \geq 0.05$).

Table 3. Results from trial 2. Effects of cecal challenge with 80,000 XN-treated *E. tenella* SZ per chick on weight gains and gross lesion scores at 6 days postchallenge.[A]

SZ treatment group	XN (ppm)	Chicks per group	Gross lesion score[BC]	Gain[BC] (g)
Untreated/unchallenged	0	5	0 ± 0 b	129 ± 5 a
Untreated/challenged	0	5	2.8 ± 0.7 a	113 ± 7 b
Treated	20	5	0 ± 0 b	130 ± 2 a
Treated	10	5	0 ± 0 b	134 ± 3 a
Treated	5	5	0.2 ± 0.2 b	137 ± 4 a
Treated	1	5	3.2 ± 0.4 a	121 ± 4 ab

[A]ppm = parts per million; SZ = sporozoites; XN = xanthohumol.

[B]Values are means ± standard error of the mean (SEM).

[C]Within columns, means with no common letter, differ significantly ($P \geq 0.05$).

***In vitro* cell invasion by *E. tenella* SZ.** For this study, *E. tenella* SZ were treated with 22 ppm XN for 1 hr. Following treatment, control and XN treatment solutions were removed from SZ by centrifugation and a wash with HBSS. The treated and washed SZ were then suspended in minimum essential medium (MEM) culture medium (American Type Culture Collection [ATCC] 30-2003) containing 5% fetal bovine serum. SZ in 1-ml aliquots were added to Madin–Darby bovine kidney (MDBK) cells grown to approximately 60% confluency on poly-L-lysine–coated coverslips in 24-well plates. Plates were incubated for 2 hr at 41 C in an atmosphere of 5% CO_2. Culture fluids, which included any free SZ, dead or alive, were then removed, cell layers were washed once with sterile phosphate-buffered saline (PBS), and coverslips were fixed for 15 min with ice-cold methanol and air-dried. Coverslips were stained with the HEMA-3 stain system (22-122911, Fisher Scientific, Hampton, NJ), air-dried, and mounted in Permount (Fisher Scientific) on glass slides. These slides were examined at 1000× (oil immersion) using a Zeiss Axioskop 2 Plus microscope. SZ within cells and outside cells were counted in 50 randomly picked fields from three coverslips, and the percentage of SZ that had invaded was calculated from the total observed (Table 1).

Assessment of XN treatment on infectivity of *E. tenella* SZ in chickens. *Trial 1.* SZ of *E. tenella*, at a concentration of 80,000/ml, were treated with 20, 2, 0.2, 0.02, or 0 (control) ppm XN as described above, washed, and resuspended in HBBS at the original concentrations. To produce challenge infections, 1-ml aliquots were administered rectally (9) to groups of five 3-wk-old chicks using a syringe attached to a ball-tipped feeding needle, with the needle tip inserted just past the ileocecal junction. The chickens were taken off feed for about 19 hr before the treatment. They were kept off feed for 1 hr after dosing, then allowed free access to feed and water. At 6 days postchallenge, chicks were killed, scored for gross cecal lesions (19), and weighed to determine any weight gains made during infection by comparing weights at the time of challenge and those at the termination of the experiments. (Table 2). Cecal segments were processed for histology using Carnoy's fixative and periodic acid-Schiff (PAS) stain (20).

Trial 2. This trial was conducted in a manner similar to trial 1, except that SZ were treated with 20, 10, 5, 1, or 0 ppm XN, (Table 3).

Assessment of XN treatment on infectivity of *E. acervulina* SZ in chickens. SZ of *E. acervulina*, at a concentration of 11 × 10^6/ml (equivalent to about 1.4 × 10^6 sporulated oocysts per ml), were treated with 20, 10, 5, 1 or 0 ppm XN as described for *E. tenella* SZ. This preparation was carefully examined to ensure that it contained no oocysts or sporocysts. To produce challenge infections, 1-ml aliquots of

Table 4. Effects of oral challenge with 11×10^6 XN-treated *E. acervulina* SZ on weight gains, gross lesions, and oocyst output at 6 days postchallenge.[A]

SZ treatment group	XN (ppm)	Gain[BC] (g)	Gross lesion score[BC]	Oocysts[BC] per cage $\times 10^8$
Untreated/Unchallenged	0	214 ± 6 b	0 c	0 d
Untreated/Challenged	0	209 ± 11 b	2.0 ± 0 a	3.00 ± 0.34 b
Treated/Challenged	20	217 ± 2 b	1.2 ± 0.2 b	2.28 ± 0.19 c
Treated/Challenged	10	244 ± 10 a	1.2 ± 0.2 b	2.80 ± 0.10 bc
Treated/Challenged	5	225 ± 9 ab	1.2 ± 0.2 b	2.76 ± 0.24 bc
Treated/Challenged	1	205 ± 5 b	1.4 ± 0.2 b	3.94 ± 0.21 a

[A]ppm = parts per million; SZ = sporozoites; XN = xanthohumol.
[B]Values are means ± standard error of the mean (SEM).
[C]Within columns, means with no common letter, differ significantly ($P \geq 0.05$).

SZ were administered to 3-wk-old chicks by gavage. These chickens had been taken off feed for about 19 hr and were each given 1.5 ml of an antacid alkali powder (10 g $CaCO_3$, 4.25 g $Mg_2O_8Si_3$, and 10.75 g colloidal kaolin suspended in 38 ml of water) (25) 10 min before SZ gavage. Chickens were kept off feed for 2 hr postchallenge, then allowed free access to feed and water. At 6 days postchallenge, chicks were killed, scored for gross lesions in the duodenum and upper small intestines (19), and weighed for gains made during infection. Additionally, feces collected from each cage of chickens during the 24 hr period before termination were homogenized and adjusted to specific volumes, and oocysts were enumerated in duplicate counts of duplicate aliquots.

Microscopic examination of treated SZ. Aliquots (100 μl) of treated and untreated *E. tenella* and *E. acervulina* SZ from *in vivo* SZ-infectivity experiments were pipetted onto 8-well printed slides and air-dried. Slides were stored at −70 C until they were ready to be stained; at which time, they were warmed to room temperature and processed. Some slides were stained with HEMA-3 stain and examined using a Zeiss Axioskop 2 Plus microscope at 1000× (oil immersion). Pictures were recorded digitally with a Nikon (Tokyo, Japan) DXM 1200 camera and Nikon ACT-1 version 2 software.

Assay of anticoccidial effects of xanthohumol presented as a feed additive. XN was mixed at 20 ppm with the broiler-starter ration that had been ground to a mash. This mash was fed to a group of 40 3-wk-old chickens from 3 days before challenge with coccidia through the termination of the experiment. An identical number of control chickens were provided with unsupplemented mash. At time of challenge, XN-treated and control chickens were each divided into four challenge groups (10 chicks per group, five per cage) on the basis of weight (12): 1) unchallenged controls, 2) *E. acervulina*–challenged (500,000 sporulated oocysts/chick,), 3) *E. maxima*–challenged (20,000 oocysts per chick), and 4) *E. tenella*–challenged (15,000 oocysts per chick) resulting in eight treatment groups. Challenge doses of oocysts were administered by gavage in 1-ml aliquots. At 6 days postchallenge, chicks were weighed, killed, and their intestines scored for gross lesions (19). Feed conversions (grams of feed eaten per grams of weight gained) during time of infection were calculated based on feed consumption and bird weight per cage of five birds. Total feces shed per cage of birds during the 24-hr period before termination were collected, homogenized, adjusted to specific volumes, and the total number of oocysts were enumerated from counts of duplicate aliquots.

Statistics. Data were analyzed by analysis of variance, and group means were compared using Student's *T*-test and Duncan's multiple range test (27).

RESULTS

***In vitro* cell invasion.** As seen in Table 1, treatment of *E. tenella* SZ with 22 ppm XN significantly inhibited the invasion of the SZ into MDBK cells. Untreated SZ could most frequently be found snug against the MDBK cell nuclei. Often, more than one SZ per cell could be seen. Far fewer XN-treated SZ were observed inside cells. Those that were counted as having penetrated were found close to cell edges, or perhaps, bound to the cell membranes. (Table 1)

Infectivity of *E. tenella* SZ treated with XN. *Trial 1.* At 5 days postchallenge with SZ, small amounts of blood were seen in droppings from chicks given untreated SZ and SZ treated with 0.02–2 ppm XN, indicating established infections with *E. tenella* in these groups. At 6 days posttreatment, no gross lesions could be seen in chicks given SZ treated at 20 ppm, whereas chicks given untreated SZ had mean gross lesion scores of about 2.6. Mean gross lesion scores for the other groups given SZ treated with lower levels of XN were not significantly different from those of the untreated control SZ (Table 2). None of the challenges with SZ produced significant weight-gain depressions. However, chicks challenged with SZ treated at 20 ppm had the highest mean weight gain (Table 2). Examination of cecal cross sections from these chicks confirmed the absence of parasites in the ceca of chicks challenged with SZ treated at 20 ppm.

Trial 2. In this trial, gross lesion scores were significantly reduced, and weight gains were maintained at the unchallenged control level in chickens challenged with SZ treated with 20, 10, and 5 ppm of XN compared with chickens challenged with untreated SZ (Table 3).

Infectivity of *E. acervulina* SZ treated with XN. Oral challenge with 11×10^6 untreated *E. acervulina* SZ per chick resulted in a mean gross lesion score of 2, indicating the success of this method of producing an infection. It did not, however, produce a significant decrease in weight gain during the infection period. Challenges with SZ treated with 20, 10, 5, and 1 ppm of XN resulted in significant lowering of gross lesion scores. The mean weight gain of chicks challenged with SZ treated at 10 ppm was significantly higher than for those of the unchallenged and untreated–challenge control groups. The oocyst outputs from SZ treated with 20, 10, and 5 ppm XN were reduced ($P > 0.028$ for 20 ppm treatment) compared with that from untreated SZ and SZ treated with 1 ppm XN. (Table 4).

Effects of XN treatment on SZ morphology. As seen in micrographs of HEMA-3–stained SZ (Fig. 1), the anterior ends of both *E. acervulina* and *E. tenella* SZ appear to be damaged by treatment with 20 ppm XN, and *E. acervulina* SZ appeared more irregular in shape (Fig. 2).

Effects of 20 ppm XN administering in feed on infection caused by *E. tenella*, *E. acervulina*, or *E. maxima*. In this experiment, the severity of infection varied with the species of *Eimeria*. Infections caused by *E. acervulina* and *E. tenella* were mild to moderate; those caused by *E. maxima* were severe. Challenge infections with *E. acervulina* and *E. maxima* caused significant weight-gain depression during infection, whereas challenge with *E. tenella* did not. Feeding XN at 20 ppm before and during challenge infections did not significantly affect weight-gain depression caused by *E. acervulina* or *E. maxima* challenge (Table 5). Feeding 20 ppm

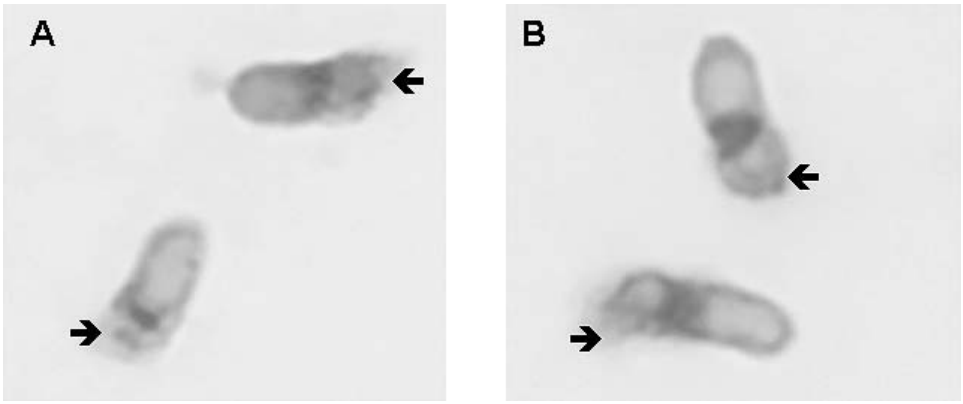

Fig. 1. Effect of treatment of *E. tenella* sporozoites with 22 ppm xanthohumol. (A) Treated *E. tenella* sporozoites. (B) Untreated sporozoites. Arrows indicate anterior ends of sporozoites.

XN only significantly reduced gross lesion scores in chickens challenged with *E. acervulina*. Feed conversions during infection were significantly increased by challenges with *E. acervulina* and *E. maxima*, but within each challenge group, no feed conversions were significantly affected by feeding 20 ppm in the mash (Table 5). In both *E. acervulina* and *E. maxima* infections, chickens fed 20 ppm XN shed numerically fewer oocysts than untreated controls during the 24-hr period before termination of the experiment.

DISCUSSION

The move to reduce the use of traditional anticoccidial drugs, as well as the widespread development of resistance to these drugs by coccidia, have prompted efforts to devise new strategies for control of avian coccidiosis. These efforts include a search for new agents with anticoccidial activity, including compounds that are naturally occurring or might be considered generally recognized as safe (GRAS).

No simple *in vitro* system exists in which to test anticoccidial activities of compounds against all life-cycle stages of *Eimeria*. However, parasite invasion of cultured cells by SZ can be studied *in vitro* (1). The ability of a substance to prevent cell invasion by a sporozoite can be one reasonable predictor of its potential for coccidiosis control. Further, only small quantities of substances are needed to conduct these assays. The initial, short-term (2 hr) invasion experiment with *E. tenella* SZ showed a definite inhibitory effect from 20 ppm XN (Table 1). Reduction in invasion appeared to be associated with physical disruption of the anterior portion of the SZ (Fig. 1). This experiment was the basis for further experiments to determine whether sporozoite treatment with XN *in vitro* could reduce parasite development *in vivo*. The difficulties of maintaining SZ viability, as determined by untreated controls, until they could reach the proper sites of host-tissue invasion was overcome by rectal inoculation with *E. tenella* SZ (9) and treatment of chicks with an antacid mixture before oral inoculation with *E. acervulina* SZ (25). These techniques showed that XN treatment of SZ was detrimental to further parasite development within the chick host.

Several preliminary tests were conducted to determine the best way to administer *E. acervulina* SZ to the chick host. The SZ did not survive when administered rectally. To overcome the problem of SZ being digested in the stomach before they could reach the duodenum, the chicks were given an antacid 10 min before dosing (25) to coat the stomach lining. Chicks were also kept off feed for 2 hr after dosing. The dose level of oocysts was calculated to be equivalent to about 1.4×10^6 sporulated oocysts, an infection dose that normally should produce severe lesions. Lower challenge doses of SZ did not produce consistent infection. Additionally, great care was taken to ensure the absence of oocysts in the SZ preparations because they might survive the digestive environment of the chick stomach and produce infection.

Weight gain was not significantly depressed by challenge with 11×10^6 untreated *E. acervulina* SZ. This challenge, based on oocyst equivalency, should have produced about a 30% weight-gain depression along with gross lesion scores of 3 to 4. The lack of significant weight-gain depression suggests that a large portion of the SZ challenge doses were lost, perhaps to digestion in the stomach, in

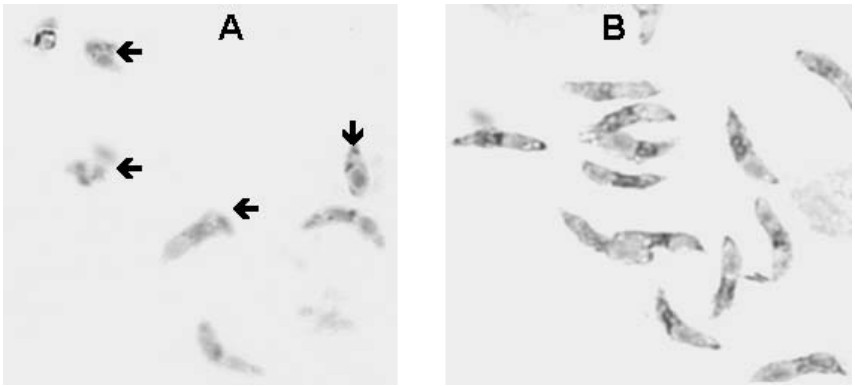

Fig. 2. Effect of treatment of *E. acervulina* sporozoites with 20 ppm xanthohumol. (A) Treated *E. acervulina* sporozoites. (B) Untreated sporozoites. Arrows indicate damaged anterior ends of sporozoites.

Table 5. Effects of 20 ppm XN fed to chickens infected with *E. tenella*, *E. acervulina*, or *E. maxima* at 6 days postchallenge ($n = 10$).[AB]

Challenge infection	XN treatment	Gain[C] (g)	Gross lesion score[C]	Feed conversion[C]	Oocyst shed per cage
None	None	143 ± 5	0	2.32 ± 0.02	0
None	XN	140 ± 3	0	2.38 ± 0.005	0
E. acervulina	None	109 ± 9	0.60 ± 0.16	2.64 ± 0.13	6.68×10^5
E. acervulina	XN	87 ± 10	0*	3.06 ± 0.14	5.43×10^5
E. maxima	None	68 ± 7	3.30 ± 0.33	4.14 ± 0.58	8.1×10^3
E. maxima	XN	78 ± 8	3.20 ± 0.29	3.42 ± 0.04	6.80×10^3
E. tenella	None	146 ± 5	1.50 ± 0.37	2.39 ± 0.06	0
E. tenella	XN	146 ± 5	1.70 ± 0.33	2.34 ± 0.09	0

[A]XN = xanthohumol.
[B]Values are means ± standard error of the mean (SEM).
[C]Significant differences between feed treatments, within each pair of challenge groups, are indicated by an asterisk.

spite of the antacid treatment. Nevertheless, this experiment shows that *in vitro* XN treatment has an apparent cidal effect on *E. acervulina* SZ because there were significant reductions in gross lesion scores and oocyst shedding from chicks challenged with treated SZ (Table 4). Obvious morphological damage was observed similar to that seen with *E. tenella* SZ (Fig. 2).

The adverse effects of XN treatment on both *E. acervulina* and *E. tenella* SZ survival and reproduction suggested that XN might be a good candidate for use as an anticoccidial feed additive. Experiments in rats by others (16,24) have shown that orally administered XN is poorly digested and remains mostly unchanged within the gut lumen. These findings indicate relatively poor absorption and subsequent concentration of ingested XN in the gut lumen, a good property for a compound targeted to intestinal parasites.

In the pilot experiment testing XN as a feed additive at 20 ppm, it was estimated that feed consumption during challenge infections averaged about 84 g/chick. Chickens eating feed containing 20 ppm XN would have consumed, therefore, about 1.7 mg XN per day. For a 500-g chicken, this would be equivalent to about 3.4 mg of XN per kg of body weight. This level of XN is apparently not sufficient to control challenge infections with the three *Eimeria* species tested. It is encouraging to note, however, that oocysts output was numerically reduced by 19% (*E. acervulina*) and 16% (*E. maxima*) in chickens consuming 20 ppm XN. Factors that may complicate an experiment such as this include the possible instability of XN within the mash, as well as within the chickens' digestive tracts. These aspects have been explored in rodents (23,24), but not in birds, and need to be addressed. The results of this pilot study point out the very long jump that needs to be taken in the practical application of *in vitro* observations.

Light micrographs indicate that XN treatment apparently disrupts the gross morphology of both *E. tenella* and *E. acervulina* SZ, particularly at the apical end, in as little as 2 hr at 40 C. The exact nature of the morphological changes is currently under investigation. Interestingly, a study on effects of a retrochalcone, licochalcone A, on another protozoan parasite, *Leishmania major*, showed by electron micrography that treatment with 10 ppm for 24 hr at 26 C destroyed the mitochondria of *L. major* promastigotes, reduced their rate of infection of human monocyte-derived macrophage (MDM), and reduced subsequent intracellular multiplication of the amastigote stage (5). This compound was also shown to have high antimalarial activity that could be demonstrated both *in vitro* and *in vivo* (6,7). XN has also been shown to inhibit growth of malarial parasites in cell culture at about 4 ppm (17,10) and to inhibit GSH-dependent hemin degradation (10). However, morphological effects of XN treatment on malarial parasites have not been reported.

Both XN and licochalcone A molecules consist of a basic chalcone nucleus substituted with a prenyl group and several hydroxyl groups. Information gained from a survey of recent literature relating molecular structure of flavonoid compounds to biological activity suggests that these structures can confer antimicrobial, antiproliferative, and cytotoxic (cancer cell lines) activities (3,8,13,14,18,21, 22,29). The prenyl side chain may also promote antimicrobial activities by imparting sufficient lipophilicity to allow xanthohumol and other prenyl chalcones and flavonoids to react with, or pass through, microbial membranes (28).

To summarize, this is the first report to demonstrate the anticoccidial activity of XN, the major prenylated chalcone found in hops flowers. It appears likely that the anticoccidial activity of XN is related to its ability to disrupt the integrity of sporozoite membranes. Importantly, XN shows activity against two of the avian *Eimeria* species during *in vitro* treatment of SZ. However, a pilot test using 20 ppm of XN mixed as a feed additive did not demonstrate good control of infections with *E. acervulina*, *E. maxima*, or *E. tenella*, as judged by the usual parameters of weight gain, feed conversion, and lesion score. Nevertheless, encouragingly, 20 ppm of XN did numerically reduce oocyst shedding in chickens infected with *E. acervulina* and *E. maxima* by approximately the same degree. To serve as a good anticoccidial feed additive, XN may have to be given at a much higher concentration. Further experiments to define proper dosage levels of XN for use as a feed additive and to explore the effects of XN on the developmental biology of *Eimeria* species are planned.

REFERENCES

1. Augustine, P. C. Cell–SZ interactions and invasion by apicomplexan parasites of the genus *Eimeria*. Int. J. Parasitol. 31:1–8. 2001.

2. Bhattacharya, S., S. Virani, M. Zavro, and G. J. Haas. Inhibition of *Streptococcus mutans* and other oral streptococci by Hop (*Humulus lupulus*) constituents. Econ. Bot. 57:118–125. 2002.

3. Botta, B., A. Vitali, P. Menendez, D. Misiti, and G. D. Monache. Prenylated flavonoids: pharmacology and biotechnology. Curr. Med. Chem. 12:713–739. 2005.

4. Casaschi, A., G. K. Maiyoh, B. K. Rubio, R. W. Li, K. Adeli, and A. G. Theriault. The chalcone xanthohumol inhibits triglyceride and apolipoprotein B secretion in HepG2 cells. J. Nutr. 134:1340–1346. 2004.

5. Chen, M., S. B. Christensen, J. Blom, E. Lemmich, L. Nadelmann, K. Fich, T. G. Theander, and A. Kharazmi. Licochalcone A, a novel antiparasitic agent with potent activity against human pathogenic protozoan species of *Leishmania*. Antimicrob. Agents Chemother. 37:2550–2556. 1993.

6. Chen, M., S. B. Christensen, T. G. Theander, and A. Kharazmi. Antileishmanial activity of licochalcone A in mice infected with *Leishmania major* and in hamsters infected with *Leishmania donovani*. Antimicrob. Agents Chemother. 38:1339–1344. 1994.

7. Chen, M., T. G. Theander, S. B. Christensen, L. Hviid, L. Zhai, and A. Kharazmi. Licochalcone A, a new antimalarial agent, inhibits the *in vitro* growth of the human malaria parasite *Plasmodium falciparum*, and protects mice from *P. yoelii* infection. Antimicrob. Agents Chemother. 38:1470–1475. 1994.

8. Di Pietro, A., G. Conseil, J. M. Perez-Victoria, G. Dayan, H. Baubichon-Cortay, D. Trompier, E. Steinfels, J.-M. Jault, H. de Wet, M. Maitrejean, M., G. Compye, A. Boumendjel, A. M. Mariotte, C. Dumontet, D. B. McIntoch, A. Goffeau, S. Castanys, F. Gamarro, and D. Barron. Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters. Review. Cell. Mol. Life Sci. 59: 307–322. 2002.

9. Fernando, M. A., M. E. Rose, and B. J. Millard. *Eimeria spp*. of the domestic fowl: the migration of SZ intra- and extra-enterically. J. Parasitol. 73:561–567. 1987.

10. Frolich, S., C. Schubert, U. Bienzle, and K. Jenett-Siems. *In vitro* antiplasmodial activity of prenylated chalcone derivatives of hops (*Humulus lupulus*) and their action with haemin. J. Antimicrob. Chemother. 55:883–887. 2005.

11. Fuller, A. L., and L. R. McDougald. Cell membrane labeling of *Eimeria tenella* SZ with the fluorescent dye PKH-67 GL for tracking parasite–host interactions. Parasitol. Res. 87:521–525. 2001.

12. Gardiner, J. L., and E. E. Wehr. Selecting experimental groups of chickens by weight. Proc. Helminthol. Soc. Wash. 17:25–26. 1950.

13. Gerhauser, C. Broad spectrum antiinfective potential of xanthohumol from hop (*Humulus lupulus* L.) in comparison with activities of other hops constituents and xanthohumol metabolites. Mol. Nutr. Food Res. 49:827–831. 2005.

14. Go, M-L., M. Liu, P. Wilairat, P. J. Rosenthal, K. J. Saliba, and K. Kirk. Antiplasmodial chalcones inhibit sorbitol-induced hemolysis of *Plasmodium falciparum*–infected erythrocytes. Antimicrob. Agents Chemother. 48:3241–3245. 2004.

15. Goto, K., T. Asai, S. Hara, I. Namatame, H. Tomoda, M. Ikemoto, and N. Oku. Enhanced antitumor activity of xanthohumol, a diacylglycerol acyltransferase inhibitor, under hypoxia. Cancer Lett. 219:215–222. 2005.

16. Hanske, L., R. Hussong, N. Frank, C. Gerhauser, M. Blaut, and A. Braune. Xanthohumol does not affect the composition of rat intestinal microbiota. Mol. Nutr. Food Res. 49:868–873. 2005.

17. Herath, W., D. Ferreira, S. I. Kahn, and I. A. Kahn. Identification and biological activity of microbial metabolites of xanthohumol. Chem. Pharm. Bull. 51:1237–1240. 2003.

18. Iwashita, K., M. Kobori, K. Yamaki, and T. Tsushida. Flavonoids inhibit cell growth, and induce apoptosis in B16 melanoma 4A5 cell. Biosci. Biotechnol. Biochem. 64:1813–1820. 2000.

19. Johnson, J., and W. M. Reid. Anticoccidial drugs: lesion scoring techniques in battery and floor pen experiments with chickens. Exp. Parasitol. 28:30–36. 1970.

20. Lillie, R. D. Histopathologic technic and practical histochemistry. 3rd ed. McGraw-Hill, New York. 1965.

21. Liu, M., P. Wilairat, S. L. Croft, A. L-C. Tan, and M.-L. Go. Structure–activity relationships of antileishmanial and antimalarial chalcones. Bioorg. Med. Chem. 11:2729–2738. 2003.

22. Miranda, C. L., Y-H. Yang, M. C. Henderson, J. F. Stevens, G. Santana-Rios, M. L. Deinzer, and D. R. Buhler. Prenylflavonoids from hops inhibit the metabolic activation of the carcinogenic heterocyclic amine 2-amino-3-methylimidazo[4, 5-f] quinoline, mediated by cDNA-expressed human CYP1A2. Drug Metab. Dispos. 28:1297–1302. 2000.

23. Nikolic, D., Y. Li, L. R. Chadwick, G. F. Pauli, and R. B. van Breeman. Metabolism of xanthohumol and isoxanthohumol, prenylated flavonoids from hops (*Humulus lupulus L.*), by human liver microsomes. J. Mass. Spectrom. 40:289–299. 2005.

24. Nookandeh, A., N. Frank, F. Steiner, R. Ellinger, B. Schneider, C. Gerhauser, and H. Becker. Xanthohumol metabolites in faeces of rats. Phytochemistry 65:561–570. 2004.

25. Rose, M. E., and P. Hesketh. Immunity to coccidiosis: Stages of the life-cycle of *Eimeria maxima* which induce, and are affected by, the response of the host. Parasitology 73:25–37. 1976.

26. Ruefer, C. E., C. Gerhauser, N. Frank, H. Becker, and S. E. Kulling. *In vitro* phase II metabolism of xanthohumol by human UDP-glucuronosyltransferases and sulfotransferases. Mol. Nutr. Food Res. 49:851–856. 2005.

27. SAS Institute, Inc. SAS/STAT user's guide, ver. 6, 4th ed., vol. 2. SAS Institute, Inc. Cary, NC. 1990.

28. Stevens, J. F., C. L. Miranda, D. R. Buhler, and M. L. Deinzer. Chemistry and biology of hop flavonoids. J. Am. Soc. Brew. Chem. 56:136–145. 1998.

29. Stevens, J. F., and J. E. Page. Xanthohumol and related prenylflavonoids from hops and beer: to your health! Phytochemistry 65:1317–1330. 2004.

30. Tabata, N., M. Ito, H. Tomoda, and S. Omura. Xanthohumols, diacylglycerol, acyltransferase inhibitors from *Humulus lupulus*. Phytochemistry 46:683–687. 1997.

31. Zhao, F., H. Nozawa, A. Daikonnya, K. Kondo, and S. Kitanaka. Inhibitors of nitric oxide production from hops (*Humulus lupulus* L.). Biol. Pharm. Bull. 26:61–65. 2003.

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